*J. Physiol.* (1986), **376**, *pp.* 163–182
*With* 13 *text-figures*
*Printed in Great Britain*



# OSCILLATORY PROPERTIES OF GUINEA-PIG INFERIOR OLIVARY NEURONES AND THEIR PHARMACOLOGICAL MODULATION: AN *IN VITRO* STUDY

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(*Received 4 June 1985*)

SUMMARY

1. The oscillatory properties of the membrane potential in inferior olivary neurones were studied in guinea-pig brain-stem slices maintained *in vitro*.

2. Intracellular double-ramp current injection at frequencies of 1–20 Hz revealed that inferior olivary neurones tend to fire at two preferred frequencies: 3–6 Hz when the cells were actively depolarized (resting potential less than −50 mV), and 9–12 Hz when they were actively hyperpolarized (resting potential more than −75 mV).

3. In 10% of the experiments spontaneous subthreshold oscillations of the membrane potential were observed. These oscillations, which resembled sinusoidal wave forms and had a frequency of 4–6 Hz and an amplitude of 5–10 mV, occurred synchronously in all cells tested within the slice.

4. These oscillations persisted in the presence of $10^{-4}$ M-tetrodotoxin and were blocked by $Ca^{2+}$ conductance blockers or by the removal of $Ca^{2+}$ from the bathing solution. The oscillations were affected by gross extracellular stimulation of the slice but not by intracellular activation of any given neurone. The data indicate that these oscillations reflect the properties of neuronal ensembles comprised of a large number of coupled elements.

5. Similar ensemble oscillation could be induced, in most experiments, by adding harmaline (0·1 mg/ml) and serotonin ($10^{-4}$ M) to the bath and could be blocked by bath addition of noradrenaline. Harmaline was found to increase cell excitability by hyperpolarizing the neurones and shifting the inactivation curve for the somatic $Ca^{2+}$ spike to a more positive membrane potential level.

6. The role inferior olivary oscillations play in the organization of motor co-ordination is discussed.

## INTRODUCTION

The electrophysiological properties of inferior olivary (i.o.) neurones have been studied with intracellular recording methods in both *in vivo* and *in vitro* conditions (Crill, 1970; Llinás, Baker & Sotelo, 1974; Llinás & Yarom, 1981*a*, *b*). Early *in vivo*

electrophysiological analysis of the i.o. demonstrated that the neurones in this nucleus tend to fire at a frequency of 1–10 Hz (Armstrong, Eccles, Harvey & Matthews, 1968) and may exhibit oscillatory firing both spontaneously (Armstrong *et al.* 1968) and following pharmacological treatment (de Montigny & Lamarre, 1973; Llinás & Volkind, 1973). Since i.o. axons project to Purkinje cells as the climbing fibre afferent system (Szentágothai & Rajkovits, 1959; Eccles, Llinás & Sasaki, 1966), the recording of complex spikes at the Purkinje cell level gives an accurate description of the electrical activity of i.o. neurones. Thus, a number of observations indicating low firing rates in i.o. neurones have been made at the level of the cerebellar cortex (Bell & Grimm, 1969; Bell & Kawasaki, 1972; Armstrong & Rawson, 1979). Indeed, at the Purkinje cell level climbing fibre activation seldom exceeds 10 Hz and the average spontaneous frequency is close to 3 Hz (cf. Llinás & Simpson, 1981).

In the present study the oscillatory properties of i.o. cells were studied *in vitro*. Some of the drugs known to enhance or modify oscillatory behaviour (such as harmaline, serotonin, noradrenaline and dopamine; cf. Sjölund, Wiklund & Björklund, 1980) were used to explore further how the ionic conductances present in the i.o. neurone interlock to generate single cell oscillatory properties. In addition, this approach also revealed how such potentials may reflect upon the mass oscillation observed both *in vivo* and *in vitro*.

## METHODS

The procedures used in this set of experiments are similar to those utilized in previous research in this nucleus (Llinás & Yarom, 1981*a*, *b*). Intracellular recordings were obtained using a bridge amplifier (Neurodata Model IR283) which allows a single or dual intracellular recording using two independent head stages. The spontaneous oscillations of the membrane potential in Fig. 4 were averaged using a Model 4094 Nicolet instrument. Extracellular stimulation was accomplished via the exposed tips of a twisted pair of Teflon-coated stainless-steel wires positioned on the surface of the slice. Current pulses of 0·05–0·1 ms and up to 500 nA were utilized in these experiments. Harmaline (0·1 mg/ml), serotonin, dopamine and noradrenaline (at final concentrations of $10^{-4}$ M) were added to the superfusion fluid and allowed to flow freely through the recording chamber. Lissajous figures in Figs. 4 and 9 were obtained by driving the time axis of the cathode ray oscilloscope with a Wavetek generator in the sinusoidal mode. The data base for this study consisted of 150 intracellularly recorded cells. Ten pairs of cells were successfully recorded in three different preparations.

## RESULTS

### *Firing properties of i.o. neurones tested by double-ramp stimulation*

Previous results have shown that i.o. neurones are endowed with voltage and $Ca^{2+}$-dependent ionic conductances which confer oscillatory properties on to these neurones (Llinás & Yarom, 1981*a*, *b*). In order to study such properties in more detail, repetitive double-ramp currents were injected through the recording micro-electrode. We hoped to determine whether single i.o. cells have preferred triggering frequencies and whether these frequencies are dependent on the resting membrane potential; this parameter is known to alter the integrative properties of these cells (R. Llinás & Y. Yarom, unpublished observations). Double-ramp current injections at frequencies of 1–20 Hz and amplitudes ranging from 0·2 to 2 mA were used to test excitability

of the i.o. neurones. Of this continuum two ranges of frequency were found which activated these neurones in a preferential manner: 3–6 and 9–12 Hz depending on the cell. These two frequencies will be referred to as the 'resonant' frequencies of these cells.

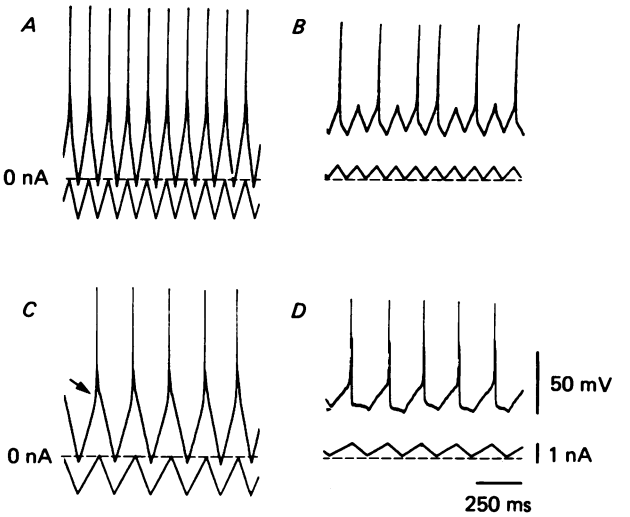


Fig. 1. Intracellular recordings from an olivary neurone during cell activation by sets of double-ramp current injection at two different membrane potential levels. The frequencies of the current ramps were 9 Hz (*A*, *B*) and 5 Hz (*C*, *D*), and the membrane potentials −78 mV (*A*, *C*) and −48 mV (*B*, *D*). Note that in *A* the cell fired at each peak but not in *B*. Also in *C* and *D* the neurone responded to the current injection at both levels of membrane potentials; however, at hyperpolarizing level (*C*), the generation of action potentials preceded the peak current (arrow). Null current level is indicated for all records (0 nA).

The results in Fig. 1 illustrate data obtained at the two 'resonant' frequencies and at two specific membrane potential levels (−78 and −48 mV). When stimulated with a double ramp at 9 Hz near the resting potential (−60 mV), i.o. cells responded with a one-to-one firing ratio (not shown). In this case, the low-threshold somatic $Ca^{2+}$ conductance, which is normally inactivated at this membrane potential, de-inactivated during the hyperpolarizing phase of the ramp and generated a rebound $Ca^{2+}$-dependent spike which triggered full action potentials (Llinás & Yarom, 1981*a*). When the cell was held at −78 mV (Fig. 1*A*), the $Ca^{2+}$-dependent somatic action potentials (arrow in Fig. 1*A*) had a lower threshold than when the ramp was introduced at resting potential level. These somatic $Ca^{2+}$ spikes were phase-locked, and in synchrony with the peak of the current ramp. By contrast, if the membrane was depolarized such that the somatic $Ca^{2+}$ conductance was not sufficiently de-inactivated (by the hyperpolarizing phase of the ramp) to generate a rebound $Ca^{2+}$ spike, the cell did not respond with full action potentials at 9 Hz but rather at a frequency close to 5 Hz (i.e. every second ramp).

Stimulation with a 5 Hz double ramp is illustrated in Fig. 1*C* and *D*. The stimulus produced a response at holding potentials both depolarized (Fig. 1*D*) and hyperpolarized (Fig. 1*C*) with respect to the rest potential. In the hyperpolarized

condition, the response always appeared during the upswing of the ramp rather than at the peak (see arrow). However, when the cell was depolarized, the action potential occurred at the peak of the voltage transient at frequencies of 2–4 Hz. Ramp stimuli below this frequency produced firing at points other than the voltage peaks.

The range of responses generated by double-ramp current injection of different frequencies is illustrated in Fig. 2 for another neurone. In this case the current

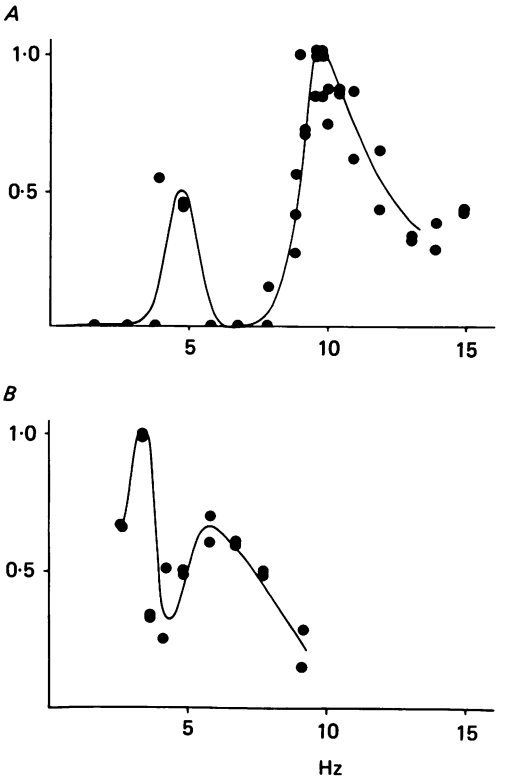


Fig. 2. Normalized firing frequency of i.o. neurones as a function of the frequency of double-ramp current injection of constant amplitude. Peak-to-peak amplitude of the double ramp was 0·25 nA. The average firing frequency was directly determined and then divided by the stimulus frequency. Membrane potentials: −72 mV (*A*) and −52 mV (*B*). The 'resonant frequency' in this cell was 9·5 Hz for the hyperpolarized level and 3·3 Hz for the depolarized level.

injection was delivered as sets of short trains 700–1000 ms in duration having a constant peak-to-peak amplitude (0·25 nA). The frequency of the double ramp was varied from 2 to 15 Hz and the average firing frequency of the impaled neurone was calculated by dividing the number of action potentials by the stimulus duration. This value was then normalized for each stimulus frequency and plotted as a function of the stimulus frequency. In this form a value of 1 indicates that the neurone generated an action potential at each depolarizing peak of the triangular wave form. Normalized response obtained at two different membrane potentials, −52 mV (Fig. 2*B*) and −72 mV (Fig. 2*A*), demonstrated the two resonant frequencies. At −72 mV the cell

followed at a frequency of 9·5 Hz, and at −52 mV the peak response occurred at 3·3 Hz. Note that both curves display secondary peaks at 5 and 6 Hz for hyperpolarizing and depolarizing levels respectively. It is likely that these peaks, which are exactly half and twice the resonant frequencies, represent harmonic resonances. We would like to emphasize that these curves were obtained at the specified holding potentials. Changing this parameter resulted in a variation of this pattern. For

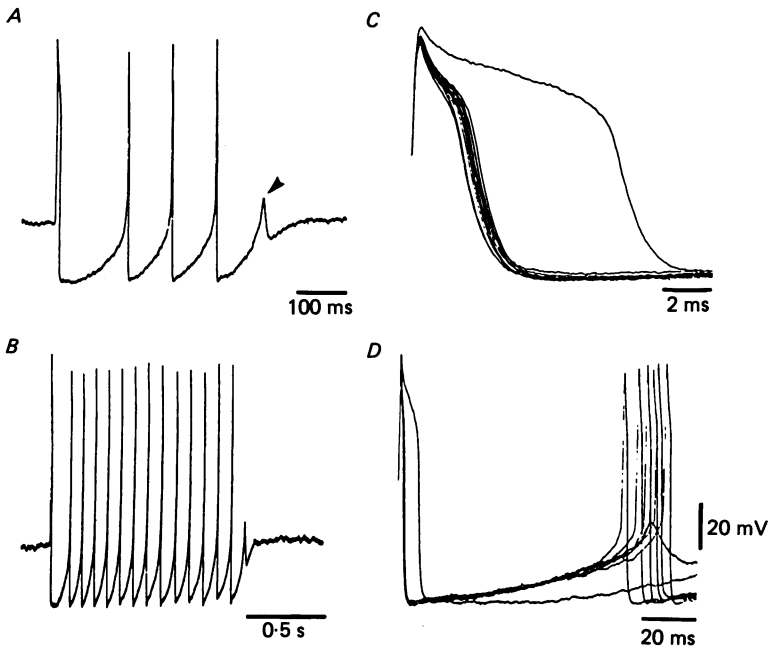


Fig. 3. Spontaneous bursts of spikes recorded intracellularly from an olivary neurone and displayed at different sweep speeds. In *A* the neurone fired four action potentials, the fifth (arrow) corresponding to a subthreshold somatic $Ca^{2+}$ spike. In *B*, a longer burst of spikes is shown at slower sweep speed. Note that the first spike interval in the burst was longer than the rest. In *C*, the action potentials shown in *B* are superimposed at a faster sweep speed. The first action potential which arises from the resting membrane potential level had a slightly higher amplitude and a rather prolonged after-depolarization which was followed (in *A*, *B*, *D*) by a prolonged after-hyperpolarization. The rest of the spikes in the train became progressively shorter until failure of spike generation occurred and the burst terminated. In *D*, the same set of records as in *B* showing the somatic $Ca^{2+}$ spike arising from the after-hyperpolarization and the range of spike intervals in the train.

example, decreasing the membrane potential to a value of −65 mV resulted in a broadening of the peaks in Fig. 2, indicating that under such circumstances the neurone may respond over a wider range of frequencies.

*Spontaneous spike bursts in i.o. neurones*

Independent evidence for the existence of these two resonant frequencies was obtained by examining the firing properties of spontaneous bursts of spikes in i.o. neurones. A set of such events is shown in Fig. 3. Fig. 3*A* shows a burst consisting

of four spikes and a rebound $Ca^{2+}$ spike (which does not reach threshold for $Na^{+}$ spike activation) (arrow). Note that the first action potential, which produces the largest after-depolarization (a $Ca^{2+}$-dependent dendritic spike (Llinás & Yarom, 1981*a*, *b*)) also produces the largest and longest lasting after-hyperpolarization (Fig. 3*A*, *B*, *D*) which results in an interspike interval of 155 ms and an instantaneous frequency of approximately 6 Hz. This initial spike is followed by a set of action potentials having a firing rate of close to 10 Hz. Another example from the same cell is shown in Fig. 3*B*. Here the burst lasted for a longer period, again showing the 6 and 10 Hz rhythm. The after-depolarization of the initial spikes and of those which follow is illustrated in Fig. 3*C* and *D* at two different sweep speeds. Here the action potentials triggered the oscilloscope demonstrating the large difference in the duration of the after-depolarization of the first spike in the burst and those that follow (Fig. 3*C*). Note also that the duration of the after-depolarization, which denotes a certain degree of dendritic invasion, subsides as the burst develops. The duration of the interspike intervals is illustrated in Fig. 3*D* and shows the difference in the duration of the after-hyperpolarization and the time of onset of the rebound spikes.

From the above, it seems evident that individual i.o. cells can resonate with two main limit cycles, one near 10 Hz (9–12 Hz) and the other near 4 Hz (3–6 Hz). The prominence of each is governed by the resting potential of the neurone and, thus, by the excitability bias toward dendritic firing when the cell is depolarized, or towards a somatic firing when the cell is hyperpolarized.

*Spontaneous ensemble oscillatory properties of the i.o. neurones*

In about 10% of the experiments, and for reasons so far undetermined, the membrane potential of all impaled neurones in particular slices showed a subthreshold oscillatory rhythm (Fig. 4*A*). Characteristically this rhythm resembled a sinusoidal wave form with a frequency of 4–6 Hz (average of $5 \pm 0{\cdot}8$ Hz, $n = 25$) and an amplitude of 5–10 mV peak-to-peak (average $8 \pm 2{\cdot}1$ mV, $n = 25$) which was, in addition, tetrodotoxin (TTX) insensitive but was abolished by addition of $Ca^{2+}$ channel blockers such as $Cd^{2+}$ or $Co^{2+}$ to the bathing medium. The regularity of these spontaneous oscillations is illustrated in Fig. 4*B* where a sequence of sinusoidal membrane potential fluctuations is displayed as a Lissajous figure. This figure was generated by feeding the voltage recorded from the cell into the $x$ axis, and a 4 Hz sinusoidal wave form into the $y$ axis of the cathode ray tube.

The spatial distribution of this oscillatory state was tested by recording simultaneously from pairs of cells (ten pairs were recorded). The distance between the cells in each pair ranged between 50 and 300 $\mu$m. One example of such a cell pair is shown in Fig. 4*C* and *D*. While in this cell passing current across its membrane did not produce a measurable voltage change in the other, this apparent lack of a demonstrable coupling is to be expected due to current spread over the cell ensemble. Nevertheless, the oscillations seen in these two cells (*a* and *b*) demonstrated the same frequency and phase and similar amplitude (see Fig. 4*C*). The average of six traces in Fig. 4*D* (*a* and *b*), shows the regularity of the response in each of the two neurones. In the last trace (Fig. 4*D*) the oscillatory responses of the two cells are superimposed. Note that their wave forms are well matched in amplitude, frequency (6 Hz) and phase. Similar records obtained from the other i.o. pairs suggest that the spontaneous

oscillatory activity includes a large number, if not most, of the cells in a given slice, and, yet, in no case was there any hint that a 'pace-maker neurone' was present in the ensemble. This was further corroborated by recording from successive cells in a slice. In some cases as many as fifteen cells were successively recorded from a given slice, all showing almost exactly the same oscillation.

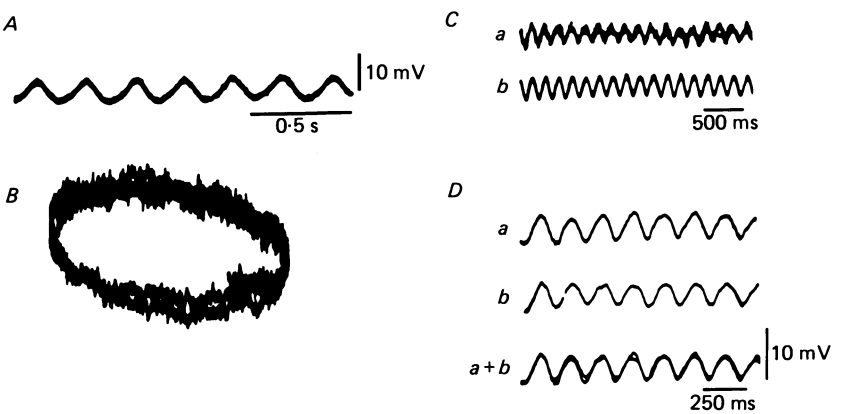


Fig. 4. Spontaneous oscillation of the membrane potential and its synchronicity in two i.o. neurones simultaneously recorded intracellularly. *A*, spontaneous oscillatory property of the membrane potential. *B*, Lissajous figure to illustrate the regularity of the spontaneous oscillation. The *x* axis of the oscilloscope was derived by a sinusoidal wave form of 4 Hz. *C*, superimposed traces of spontaneous membrane potential oscillations recorded simultaneously from two olivary neurones (*a*, *b*). *D*, an averaged record of six traces recorded during the same time interval from the two cells in *B* and superimposed in *a* and *b*. Note that the membrane potential of both neurones oscillated in phase and with similar amplitude. Records taken in the presence of TTX in the bathing solution.

The regularity of these spontaneous oscillations can also be seen in Fig. 5*A–C*. Upon membrane depolarization, dendritic $Ca^{2+}$ spikes (truncated) were evoked (Fig. 5*A*); whereas, when the membrane was hyperpolarized, somatic $Ca^{2+}$ spikes could be observed (Fig. 5*C*). Both somatic and dendritic action potentials occurred during the depolarizing phase of the oscillation; however, the spikes did not necessarily occur in every cycle. Note also that the frequency of the ensemble oscillation was not modified by membrane potential, the only observable change being a small reduction in amplitude which occurred both upon depolarizing and hyperpolarizing the membrane in a range sufficient to activate delayed and anomalous rectification.

In another preparation (Fig. 5*D*), the spontaneous oscillatory rhythm generated somatic $Ca^{2+}$ spiking when the cell was hyperpolarized by 5 mV. This 6 Hz oscillation was unchanged by addition of TTX to the bath at a concentration of $10^{-5}$ M. While somatic $Ca^{2+}$ spiking generally occurred on the upswing of each of the oscillations, on occasion a shift in the onset of the somatic $Ca^{2+}$ spikes could occur as illustrated in Fig. 5*D*. This shift is even clearer in Fig. 5*E* after the membrane was hyperpolarized by 10 mV and firing of the cell occurred 180 deg out of phase with respect to the basic oscillation. This out-of-phase firing could continue and, at some point, the spike would

skip a cycle and go back into phase once again. Results such as these indicate that if a given neurone fires out of step, it may remain so only if the phase shift is close to 180 deg with respect to the original oscillations. At points closer to the main cycle the action potentials fall within the oscillatory frequency and phase by being either advanced or delayed with respect to the basic rhythm. These results also indicate that the ensemble oscillatory properties of given cells are separable from those of the

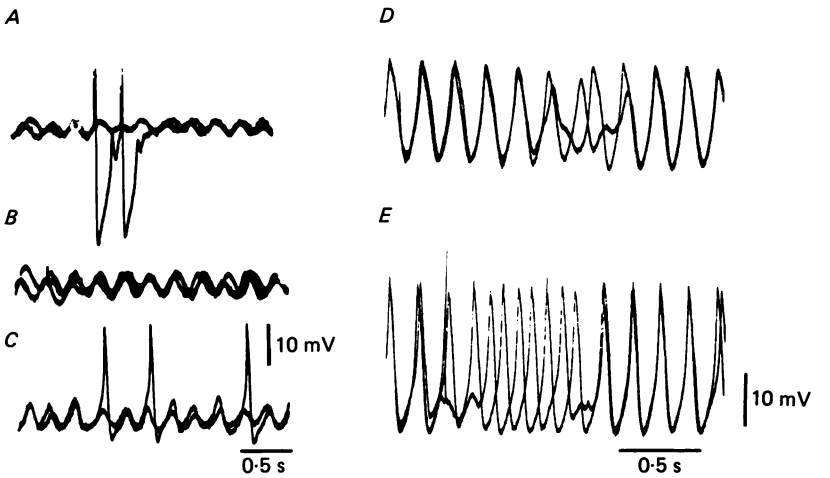


Fig. 5. Separation of single cell excitability from the subthreshold oscillatory property. *A–C*, superimposed traces of membrane potential at rest (*B*), at 15 mV depolarization (*A*) and at 24 mV hyperpolarization (*C*). Note that while the spontaneous oscillations may trigger dendritic (*A*) and somatic (*C*) $Ca^{2+}$ spikes, the frequency of the oscillation was not modified by the change in membrane potential. *D*, two superimposed traces of spontaneous oscillations at two levels of hyperpolarization. Upper panel at −5 mV and lower at −10 mV from rest level. Each wave of oscillation triggers a somatic $Ca^{2+}$ response. In the upper trace three spikes occur out of step with respect to the oscillations. In the lower trace the somatic spike is larger, and out of step firing occurred for four cycles. Records taken with TTX in the bath.

spontaneous ensemble oscillation since individual spikes can occur as separate phenomena, and since the ensemble oscillations are themselves not very susceptible to changes in membrane potential.

*Effects of intracellular and extracellular stimulation on spontaneous ensemble oscillation*

Given the above spontaneous ensemble oscillatory state, it was considered whether such state could be gated or otherwise modified by direct electrical stimulation capable of activating a large portion of the slice or a single neurone. To this effect, two stimulus paradigms were utilized, gross extracellular stimulation and intracellular stimulation. Extracellular stimuli capable of activating a large proportion of the cells were delivered to the surface of the brain-stem slice via a pair of metal electrodes placed at the centre of the i.o. nucleus. The results of such an experiment are illustrated in Fig. 6. Intracellular recordings are displayed at a slow sweep speed in

Fig. 6*A* where the external stimuli are shown to generate a full action potential for each of the stimuli (delivered at 5 Hz, Fig. 6*B*). The results demonstrate that if the cell was oscillating at the time the stimulus was begun (arrowhead), the rhythmic activity would stop. Furthermore, this oscillatory rhythm could be quenched beyond the duration of the stimulus train, and was accompanied by a membrane hyperpolarization (see dashed line in Fig. 6*A*). After about 4 s, following the termination of the stimulus, the membrane potential returned to rest value slowly and again showed signs of an ensemble oscillatory rhythm which returned to the control level

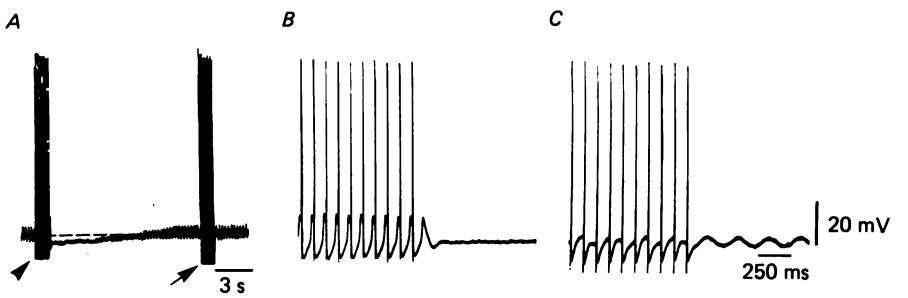


Fig. 6. Transient annihilation of the subthreshold oscillation by extracellular electrical stimulation. *A*, continuous recording of spontaneous oscillations interrupted by a brief train of action potentials generated by either extra- (arrowhead) or intracellular (arrow) stimulus. The extra- and intracellularly evoked action potentials are shown at faster speed in *B* and *C* respectively. Following extracellular stimulation(*A*, *B*) the oscillation of the membrane potential disappears for 4 s. This does not occur following single cell stimulation (*A*, *C*).

approximately 7 s after the stimulation. This type of response could be obtained repeatedly in a given cell and was observed in all cases tested.

In contrast to the above, direct stimulation of the cell via the recording electrode (arrow, Fig. 6*A*), at the same frequency utilized for the extracellular stimulus, elicited direct firing of the cell but did not modify the oscillatory rhythm (Fig. 6*C*). This finding implies that the activation of a single cell is incapable of resetting the rhythm of the ensemble.

*Harmaline and its action on the oscillatory rhythm*

Addition of harmaline to the bath in a final concentration of 0·1 mg/ml produces a clear effect on the anomalous rectification (R. Llinás & Y. Yarom, unpublished observations) and, in addition, it hyperpolarizes the membrane and increases the somatic $Ca^{2+}$ spike amplitude. This increase in somatic $Ca^{2+}$ spike comes about by hyperpolarizing the cell as well as by a direct action of harmaline on the rebound $Ca^{2+}$ conductance.

The action of harmaline on membrane potential is illustrated in Fig. 7. At the arrow, harmaline was added to the bath while the excitability of the cell was continuously monitored using square current pulses which were subthreshold at the beginning of the recording. The records in Fig. 7*A* show the time course of the hyperpolarization and the increased excitability of the neurone following the addition of the drug. In

Fig. 7*B* single records taken from the sequence shown in *A* are displayed at a faster sweep speed to show in detail the gradual increase in excitability after harmaline administration. Thus, the current pulse, subthreshold in 1, generated a small response in 2, a larger somatic $Ca^{2+}$ action potential in 3, and in 4 a full $Ca^{2+}$ action potential which activated a fast $Na^{+}$-dependent spike. As the membrane continued to hyperpolarize, the pulse became subthreshold once again in the sense of not reaching the firing level for the somatic $Ca^{2+}$ spike.

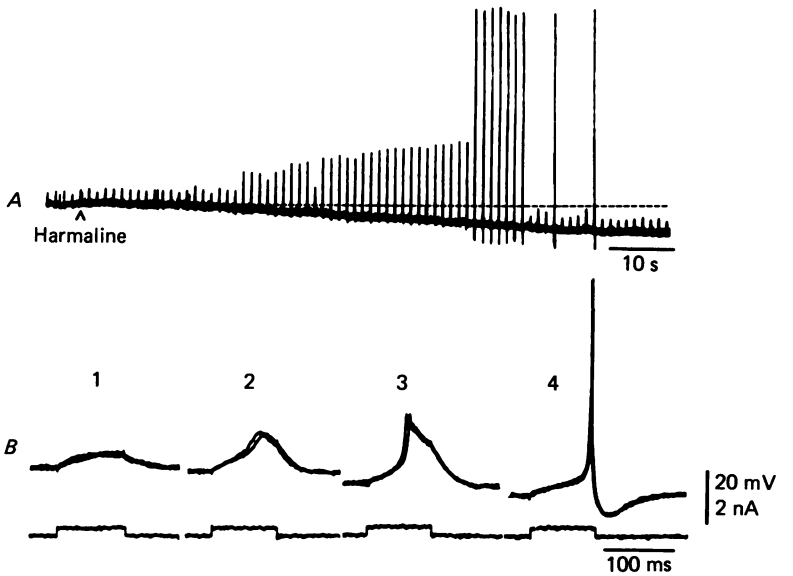


Fig. 7. Hyperpolarization and increased excitability induced by harmaline. A set of constant amplitude depolarizing current pulses were delivered at constant intervals and displayed at slow and fast sweep speed in *A* and *B* respectively. Harmaline ($10^{-5}$ M) was added at arrow. The superimposed traces in *B* were taken at different times after the addition of the drug. Note that after the addition of harmaline the membrane potential gradually increased and subthreshold current pulses generated action potentials. As the hyperpolarization progressed, the pulses once again became subthreshold.

The possibility of a direct effect of harmaline on the somatic $Ca^{2+}$ conductance was studied by measuring the first derivative of the somatic $Ca^{2+}$ action potential at different levels of membrane potential following TTX poisoning. As in a previous paper (Llinás & Yarom, 1981*b*), the level of $Ca^{2+}$ excitability was determined by the rate of rise of the somatic spike at different levels of membrane potential using uniform stimulation. An action potential recorded before harmaline is shown in Fig. 8*A* and the action potential generated by the same current pulse and from the same membrane potential following harmaline administration is illustrated in Fig. 8*B* (the first derivative is shown in the lower records). Measurements of the rate of rise as a function of membrane potential are plotted in Fig. 8*C* for control (circles) and harmaline (triangles) data. The plot indicates that after harmaline, a hyperpolarization of 18 mV was sufficient to completely de-inactivate the $Ca^{2+}$ conductance while under normal conditions even 25 mV of hyperpolarization did not achieve a full de-

inactivation. However, the plot of the rate of rise has a shape similar to that seen prior to the addition of harmaline, suggesting that harmaline has a direct effect on the somatic $Ca^{2+}$ spike.

The effects of harmaline on a spontaneously oscillating neurone are demonstrated in Fig. 9. Following addition of harmaline to the bath (Fig. 9*A*), the cell was

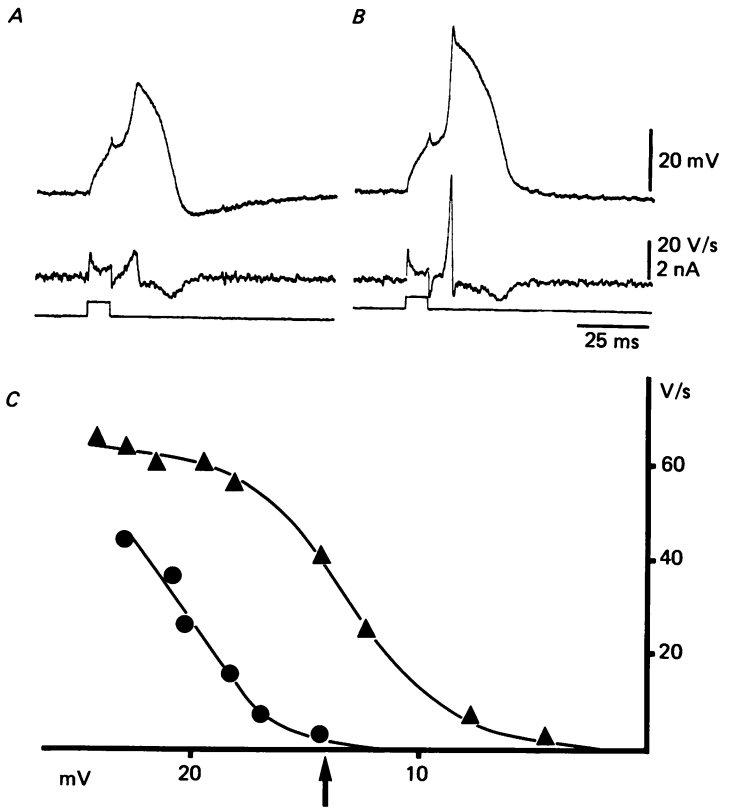


Fig. 8. Effect of harmaline on the voltage dependence of the somatic $Ca^{2+}$ spike. *A* and *B*, somatic $Ca^{2+}$ spikes generated by short current pulses (lower traces) from a constant holding potential (−17 mV below rest), before (*A*) and after (*B*) the addition of harmaline. *C*, maximum rate of rise of the somatic $Ca^{2+}$ spike, measured from the voltage derivative (middle traces), as a function of the holding potential, before (circles) and after (triangles) the addition of harmaline. Arrow indicates the resting potential after addition of harmaline. Records obtained in the presence of TTX.

hyperpolarized and the somatic $Ca^{2+}$ spikes increased in amplitude until full action potentials were generated; although in some cases the hyperpolarization was large enough to prevent the somatic $Ca^{2+}$ spike from reaching the firing level for $Na^{+}$ spike activation. The three sets of superimposed traces in Fig. 9*B* were triggered by the oscillatory rhythm and show the regularity of the response and the progressive effect of harmaline. In this particular case the frequency of oscillation was close to 6 Hz. In *C*, *D* and *E* of Fig. 9 the records shown in *B* are displayed as Lissajous figures

generated by 6 Hz double-ramp voltage function. The present results demonstrate, once again, the regularity of i.o. oscillation even during the increased excitability generated by the progressive action of harmaline. Note that while the oscillations are regular throughout, a slight change in the frequency is apparent. Thus prior to harmaline the oscillatory frequency was 5·3 Hz. As the somatic spiking became dominant, this frequency increased to 6·1 Hz.

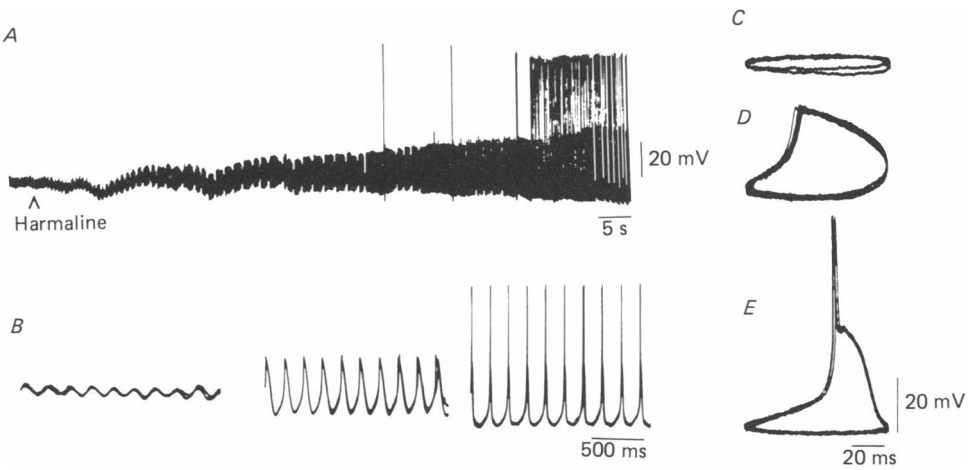


Fig. 9. Harmaline-induced rhythmic firing in a spontaneously oscillating neurone. *A*, continuous recording of membrane potential before and after the addition of harmaline. *B*, three superimposed traces taken at different times after the addition of the drug, demonstrating the development of the somatic $Ca^{2+}$ spikes triggered by the sinusoidal oscillations. As the amplitude of the somatic $Ca^{2+}$ spikes increased, they reached threshold for $Na^{+}$-spike activation. *C*, *D*, *E*, the same as in *B*, displayed as a Lissajous figure.

The above results indicate then that harmaline has at least two separate effects on i.o. neurones: (i) it increases the voltage sensitivity of the somatic $Ca^{2+}$ conductance, and (ii) it hyperpolarizes the cell. The combination of these two effects acts to increase excitability such that single cells tend to repetitively fire at a frequency close to 10 Hz. These effects of harmaline are quite irreversible when compared to the action of the other bioactive substances utilized in this research. Indeed, following a single administration of harmaline to the bath, lasting for approximately 10 min, no sign of recovery could be seen in any of the experiments, even after 4 h of continuous wash with Ringer solution.

*Pharmacological effects on spontaneous oscillatory properties of the i.o.*

In some cases where the membrane potential of the i.o. neurones in a given slice did not demonstrate spontaneous oscillation such as illustrated in Fig. 7, harmaline could be applied to the bath without generating the ensemble oscillatory response. However, when harmaline was introduced to the bath with either serotonin or dopamine, oscillations were almost invariably observed. In this case the oscillatory rhythm was slower than that seen spontaneously *in vitro*. The results presented here are based on eighty cells studied under harmaline, of which thirty were given

serotonin and eight dopamine in addition. Twenty-five additional cells were studied under serotonin alone and fourteen under dopamine alone.

Four examples of the types of oscillatory rhythm which follow combined application of harmaline and dopamine or serotonin are shown in Fig. 10. These particular examples are representatives of the large variety of wave shapes encountered. Their frequency ranged between 0·5 and 2 Hz.

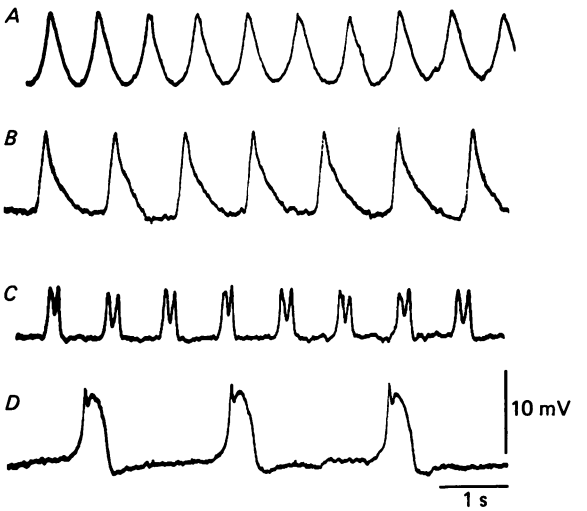


Fig. 10. Pharmacologically induced rhythmic activity in i.o. neurones. The effect of serotonin (in *A* and *C*) and dopamine (*B* and *D*) on olivary neurones pre-treated with harmaline. The records were obtained from neurones in different slices, demonstrating the variability of the responses. None of the cells displayed spontaneous activity before the treatment. TTX was added to the bath.

In contrast to harmaline which tends to hyperpolarize i.o. neurones, serotonin or dopamine depolarized these cells and increased dendritic excitation, even in the presence of harmaline. Thus, when either serotonin or dopamine were paired with harmaline, the oscillatory responses could arise from an otherwise steady membrane potential level, as shown in Fig. 10. In these cases the sinusoidal character of the response is usually lost and a more triangular shaped oscillation with a shallow negative phase is seen (Fig. 10*A*). In other cases (as in Fig. 10*B*), rather than having a triangular shape, the oscillation was characterized by a fast rising phase and a slow decay, the rising phase again coming from a rather steady background potential. Finally, as shown in Fig. 10*D*, oscillation may have a shape similar to dendritic $Ca^{2+}$ spikes with a fast rising rate and a slow after-depolarization. These patterns are not altered by bath application of TTX.

*Properties of pharmacologically evoked membrane oscillations*

Membrane potential oscillation following harmaline and serotonin are quite independent of membrane resting potential. Indeed a cell oscillating at 1 Hz can generate somatic $Ca^{2+}$ spikes at that frequency if the membrane is hyperpolarized

(Fig. 11, −0·07 nA). If artificially depolarized, dendritic spikes would also be activated at the same frequency (Fig. 11, 2·0 nA). This indicates that, as in the case of the non-pharmacologically induced spontaneous oscillation, the mechanisms responsible for the generation of the above oscillation are independent of membrane potential and spike electroresponsiveness.

The oscillatory rhythm generated by the combined application of serotonin or dopamine with harmaline was found to be modifiable, as in the case of spontaneous

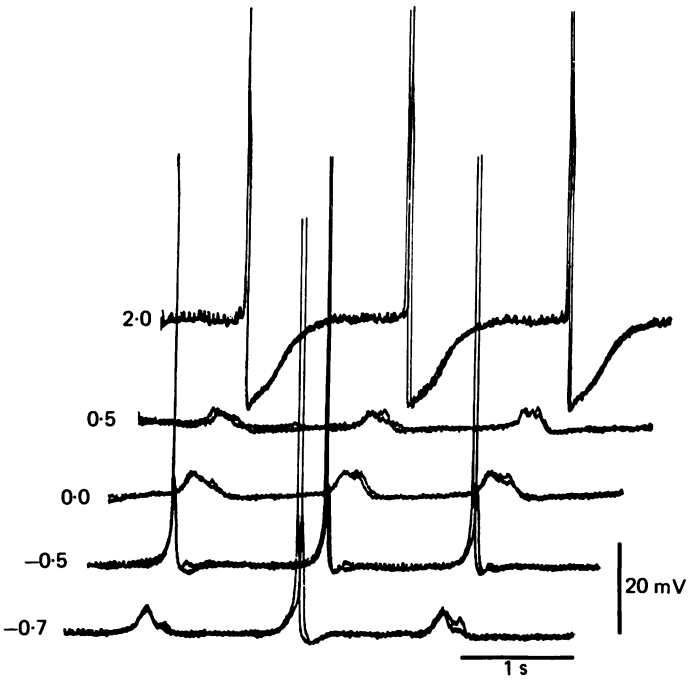


Fig. 11. Independence of oscillatory rhythm frequency on resting membrane potential. Oscillations were brought about by harmaline and serotonin. The membrane potential was shifted to hyperpolarizing and depolarizing directions by direct current injection, as indicated at the left of each trace in nA. Traces were shifted in order to avoid overlapping of the response.

oscillations, by the extracellular stimulation of the i.o. with wire electrodes. As an example, the cell illustrated in Fig. 12*A* was oscillating at a frequency of 1·5 Hz. Following three extracellular stimuli (arrows), the wave-shape of the oscillation changed and its frequency increased. Then after about 10 s, the membrane showed a continuous small depolarization and the oscillations dampened and finally stopped. This cell was monitored for 10 min with no signs of oscillations and, prior to stimulation, the oscillatory rhythm had been continuously present for 20 min. Another example, one of the few where the i.o. cells were not oscillating after the above pharmacological treatment, is shown in Fig. 12*B*. Here full stimuli (arrows) activated an oscillatory rhythm which dampened and disappeared after about 12 s. A different type of change in the oscillatory response brought about by external stimulation is shown in Fig. 12*C* where the electrical stimuli (arrows) are shown to modify the shape

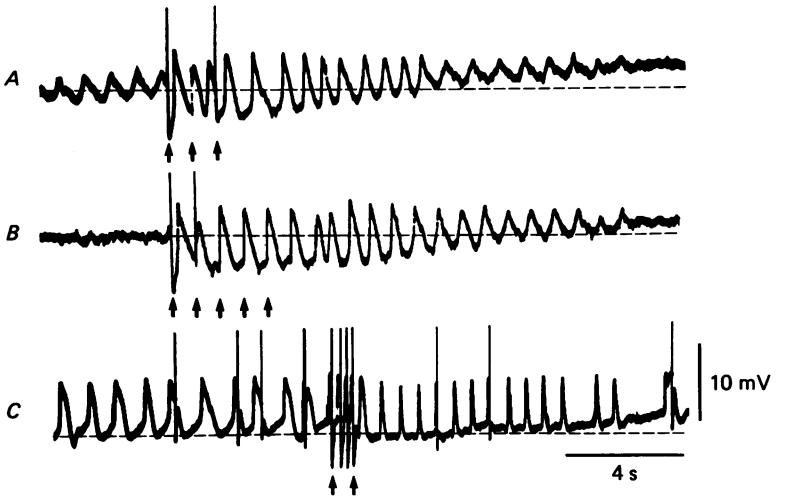


Fig. 12. Modulation of pharmacologically induced rhythmic activity in i.o. neurones by extracellular stimulus. *A–C* represent three different neurones in which the stimuli either reduced the oscillation (*A*), induced oscillation (*B*), or modified the wave form of the oscillations (*C*). All the cells were pre-treated with harmaline and serotonin. Dashed line shows deviation from the rest membrane potential.

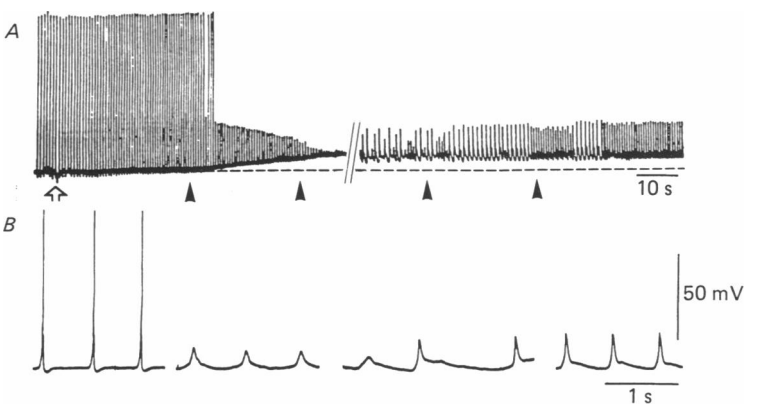


Fig. 13. Abolition of pharmacologically induced rhythmic activity by noradrenaline (NA). The neurone was pre-treated with harmaline and serotonin prior to the addition of NA ($10^{-3}$ M, 0·1 ml) to the bathing solution (arrow). Break in the record shows resumption of activity 8 min after $Na^+$ wash-out. *B*, faster sweep traces taken at different times (arrowheads) after the addition of NA, demonstrating the alterations in shape and frequency of the spontaneous activity.

and frequency of the oscillation. This modification lasted for 15 s after which the oscillatory rhythm returned to its previous stable state.

*Effect of noradrenaline on drug-induced oscillation*

The addition of noradrenaline (NA) to the bathing fluid at a concentration of $10^{-4}$ M had no obvious effect on the electrophysiology of the i.o. neurones. However, following the initiation of oscillatory behaviour by harmaline and serotonin, the

addition of NA depolarized the membrane and completely obliterated the oscillatory rhythm (Fig. 13). This was observed in each of five slices in which all cells tested prior to NA treatment demonstrated membrane potential oscillation while all cells recorded after NA lacked the oscillatory property. Abolition of the oscillatory behaviour lasted for 8–10 min after the removal of NA from the bath (break in trace, Fig. 13*A*). The rhythm recovered initially as a rather disorganized oscillation. Fig. 13*B* shows traces taken (as indicated by arrowheads) from Fig. 13*A* and displayed at a faster sweep speed.

## DISCUSSION

### *Ensemble oscillatory properties of i.o. cells*

The results described above indicate that i.o. neurones are capable of generating repetitive full-spike activation, resonating at two distinct frequencies, one ranging from 3 to 6 Hz, the other from 9 to 12 Hz. These two frequencies reflect, respectively, the predominantly dendritic or predominantly somatic distribution of the electro-responsiveness of these cells, as modulated by the resting membrane potential level. Indeed, this repetitive firing behaviour must be regarded as an expression of the spatial distribution of regenerative spike activity. In a slightly depolarized cell, neuronal firing frequency will be modulated by the degree of $Ca^{2+}$-dependent activation by the dendritic spike which, in turn, governs the size and duration of the after-hyperpolarization and thus the firing frequency. At more hyperpolarized levels the active invasion of dendrites is prevented, given their rather high threshold, and the repeated activity is dominated by the de-inactivation of the somatic $Ca^{2+}$ conductance. Excellent support for this view is provided by the recent demonstration (with patch-clamp techniques) of two distinct $Ca^{2+}$ channels having voltage activation curves which fit closely the requirement for the somatic (inactivating) and the dendritic (non-inactivating) $Ca^{2+}$ conductances (Carbone & Lux, 1984; Nowycky, Fox & Tsien, 1984*a*, *b*).

### *Sinusoidal oscillations*

The oscillatory behaviour of the membrane potential of the i.o. neurones may be considered as an emerging property arising from the membrane conductance of the individual neurones summed via the electrical coupling network that links them (Llinás *et al.* 1974; Llinás & Yarom, 1981*a*). Indeed, the synchronous sinusoidal oscillation, seen intracellularly in non-pharmacologically treated slices, seems to be generated by subthreshold properties of the i.o. neuronal ensemble.

The sequence of events that serve as the basis for this oscillation may be described as involving an inward $Ca^{2+}$ current which activates a $Ca^{2+}$-dependent $K^+$ conductance ($g_{K(Ca)}$) that in turn leads to hyperpolarization followed by a $Ca^{2+}$-dependent rebound conductance change. This conclusion is supported by the finding, that if TTX is added to the bath in sufficient concentration to block $Na^+$ action potentials, no alteration of this oscillatory behaviour is observed. However, if the $Ca^{2+}$ conductances are blocked by either $Co^{2+}$ or $Cd^{2+}$ or by replacement of $Ca^{2+}$ by $Mn^{2+}$, the oscillatory rhythm disappears.

The spontaneous oscillations are symmetrical and close to sinusoidal and are

capable of firing the cell during their depolarizing phase as either somatic or dendritic spikes, depending on the resting membrane potential level. The frequency of this oscillatory activity seems to be independent of the electroresponsive state of any given neurone in the ensemble since direct activation of the cell through the recording electrode does not alter the spontaneous rhythm. The fact that little or no difference is observable between the subthreshold oscillations of pairs of cells, as relating to amplitude, frequency or phase, further suggests that a very small amount of current is flowing between these neurones and that a state similar to a parametric oscillation (Andronow & Chaikin, 1949; Winfree, 1980), may be operant. Current flow between cells would tend to produce the uniformity of oscillation observed in the different neurones in a given slice, with the distributed electrical coupling serving as a low-pass filter (Bennett, 1977) which contributes to the sinusoidal shape of this oscillation.

The conclusion is then reached that in C.N.S. nuclei, such as the i.o., multicellular oscillatory events may take place in the absence of all-or-none electroresponsiveness. The oscillatory rhythm is then a property intrinsic to the ensemble. This ensemble interacts with electrical stimuli such that they may be enhanced or diminished by the phase of the intrinsic oscillation or, if large enough, may actually reset the oscillatory properties of the ensemble (Winfree, 1982). In this respect then the i.o. nucleus displays some of the topological properties described in the heart by the Winfree model (Winfree, 1982). Indeed, the existence of singularities may be observed; that is, on occasions a train of stimuli can totally annihilate the oscillation for a functionally significant period of time. Alternatively, repetitive activation by electrical stimulation may initiate the oscillations in an otherwise quiescent slice or may alter the basic oscillatory frequency immediately following stimulation.

*Pharmacologically triggered oscillations and their modulation by NA*

The spontaneous oscillations of the i.o. nucleus can be deeply influenced by harmaline. This drug has at least two main effects on the i.o. cell: (a) it hyperpolarizes the neurone, and (b) it has a direct effect on the low threshold conductance (see Fig. 8). These actions of harmaline facilitate the oscillatory behaviour of i.o. cells by increasing the rebound somatic $Ca^{2+}$ conductance. In cells where oscillatory response is not observed, harmaline may produce the above effects without generating ensemble oscillatory behaviour (Fig. 7).

If, on the other hand, harmaline is paired with either serotonin or dopamine (both of which depolarize the cell and increase its input resistance), the neurones will still demonstrate an increased $Ca^{2+}$ excitability. Moreover, under these circumstances, i.o. cells oscillate at a rather low frequency. This frequency depends on a set of parameters all of which have not been determined. It is not unlikely, however, that one of these parameters may be the actual number of electrically coupled neurones in a given slice. Thus, a large number of coupled neurones may result in the production of spontaneous oscillations, while with fewer coupled neurones oscillation may only be induced pharmacologically. This latter case may come about by the combined action of (a) the increased rebound $Ca^{2+}$ conductance due to harmaline, and (b) increasing the degree of coupling between neurones, due to an increased input resistance that follows serotonin or dopamine administration. In addition, this modulation of frequency may be due to a combination of intrinsic electrophysiological characteristics of the cell

membrane and an intracellular chemical oscillator capable of being modified externally by specific modulatory substances (cf. Goldbeter, Martiel & Decroly, 1984).

I.o. neurones are known to change their oscillatory behaviour in the presence of certain pharmacological substances such as barbiturates. Lamarre & Mercier (1972) reported that following administration of Nembutal, the frequency of harmaline oscillation varied from 10 to 5 Hz in a dose-dependent manner. In attempting to determine whether the oscillatory behaviour observed in the *in vitro* i.o. preparation could also be modified by substances which are known to be modulators *in vivo*, NA was added to the bath following a harmaline–serotonin oscillatory state in the expectation that it might reduce the oscillation. This was expected as NA has been shown to block $Ca^{2+}$ conductances in central neurones (Horn & McAffee, 1980; Adams & Galvan, 1981). In our case, this agent had the effect of initially increasing the frequency of the oscillation and then completely blocking it.

*Possible role of i.o. oscillation in motor coordination*

The results described in this paper indicate that i.o. neurones *in vitro* possess intrinsic mechanisms which allow them to function as a synchronized neuronal ensemble. I.o. neurones *in vivo* tend to fire at low frequencies and thus oscillations as described here were not really expected in the real animal. However, auto-correlogram and cross-correlogram studies of spontaneous or evoked climbing fibre activations using two simultaneous recordings (Bell & Kawasaki, 1972) or from sixteen or thirty-two simultaneously recorded Purkinje cells have shown clear peaks at 100 ms intervals (see Bower & Llinás, 1982, 1983; Sasaki & Llinás, 1985). This 10 Hz rhythm in the autocorrelogram is made more visible by the application of harmaline and is accompanied by tremor produced, via collateral activation of i.o. neurones to the cerebellar nuclei. These findings imply that oscillations such as have been illustrated in this paper actually occur *in vivo* and that the climbing fibre system operates as a type of pace-maker device which may modulate the onset of muscle movements. Indeed, it has been suggested that the rebound property of the individual i.o. neurones may be at the basis of the so-called physiological tremor (Llinás, 1984) which is clearly observable in man and has a frequency close to 10 Hz (Marsden, 1984). While the functional importance of a 10 Hz rhythm has been discussed in detail relating to the organization of movement in several mammals (cf. Stein & Lee, 1981), the precise organization of the olivo-cerebellar system and the manner in which such rhythm may interact with other pre-motor signals are still a matter of conjecture. It is nevertheless clear that the probability of activating specific muscle masses at a particular moment in time may be increased by the existence of a pace-maker system capable of serving as a timing signal for muscular activation.

Research was supported by United States Public Health Service grant NS13742 from the National Institute of Neurological and Communicative Disorders and Stroke.

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